As the Advisory Action is understood, claims 16-23 remain rejected under 35 U.S.C. §103 over Lill et al., alone, or in view of Poethke et al.

The rejection is traversed.

Applicants disclose and claim a method for screening a compound that inhibits or enhances activity of an acetyltransferase to catalyze a reaction that transfers an acetyl group from one substrate to another. Applicants method, as amended herein, comprises:

(a) contacting the acetyltransferase with a peptide substrate in a presence of a test compound,

(b) detecting an amount of an acetylated peptide substrate using an anti-acetylated peptide antibody, wherein the anti-acetylated peptide antibody recognizes only an acetylated form of the peptide substrate and does not recognize to any appreciable degree the peptide substrate in its unacetylated form,

(c) comparing the amount of the acetylated peptide substrate detected in step (b) with a control amount defined as an amount of an acetylated peptide substrate detected in an absence of the test compound, and

(d) selecting the compound associated with an increase or decrease in the amount of the acetylated peptide substrate as compared to the control amount.

The cited references, whether considered alone or in combination, do not teach or suggest the methods of the present invention.

For instance, the antibodies of the present invention specifically recognize an acetylated peptide and do not recognize to any appreciable degree the unacetylated form.

In contrast, the antibodies reported by Lill et al., pAb421 and pAb1801, bind to acetylated

p53 of a p53-p300/CBP complex, and also bind to unmodified p53. Indeed, these antibodies are commercially available and were raised against the C-terminal peptide and N-terminal peptide of p53, respectively (see Appendix A, attached hereto). Clearly, these antibodies bind to unmodified p53.

In support of the afore-mentioned arguments, and to further illustrate the distinctions between the cited art and the present invention, Applicants submit herewith a copy of the following literature reference: Zaika et al., *J. Biol. Chem.* 274(39): 27474-27480, 1999 (see Appendix B, attached hereto).

In particular, attention is directed to that reference at page 27475, left column, last four lines:

> Antibodies to p53 were monoclonals PAb 421, 1801, and DO-1 (Oncogene Science), which recognize epitopes amino acids 372-382 (421), 46-55 (1801), and 20-25 (DO-1).

Additional reference is made to page 27478, in the legend of Fig. 4:

> Shown is the immunofluorescence of parental and transfectant cells with PAB 1801 (left column) and 421 (right column). Both antibodies are specific for endogenous p53. PAb 421 is a modification sensitive antibody that recognizes its epitope (amino acids 372-382) in the unmodified states. Whereas PAb 1801 recognizes p53 in the cytoplasmic (parental) and nuclear (transfectant) compartment of LAN-5 cells, PAb 21 gives no signal or only a minimal signal. MDA 231 control cells are well recognized by 421. X 400.

Still further reference is made to page 27479, left column, at lines 23-24:

> Both PAb 1801 and PAb 421 antibodies are specific for endogenous p53.

In view of the aforementioned arguments, the claimed invention clearly is not rendered obvious by Lill et al.; for example, *by use of antibodies which recognize both acetylated p53 and unmodified p53*. Lill et al. do not teach or suggest antibodies that specifically recognize acetylated p53 and do not recognize to any appreciable degree the unmodified p53.

The Poethke document does not cure the deficiencies of the Lill document. Poethke merely reports an ELISA system, and does not disclose or otherwise suggest detection of an acetylated peptide substrate using an anti-acetylated peptide substrate antibody. Thus, even if the Lill et al. reference were combined with that of Poethke et al., the present invention would not have been obvious to the skilled artisan.

Section 2143.03 of the Manual of Patent Examining Procedure, states in part:

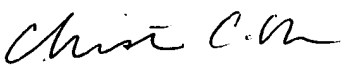
> To establish *prima facie* obviousness of a claimed invention, all the claim limitations must be taught or suggested by the prior art.

Indeed, Applicants' claimed methods include detecting an acetylated peptide substrate using an anti-acetylated peptide substrate antibody that recognizes an acetylated form of the peptide substrate and does not recognize to any appreciable degree the peptide substrate in its unacetylated form.

In view thereof, reconsideration and withdrawal of the rejections are requested.

It is believed the application is in condition for immediate allowance, which action is earnestly solicited.

Respectfully submitted,



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MARKED VERSION TO SHOW CHANGES

IN THE CLAIMS

Claims 16 and 23 were amended as follows:

16. A method for screening a compound that inhibits or enhances activity of an acetyltransferase to catalyze a reaction that transfers an acetyl group from one substrate to another, the method comprising:

(a) contacting the acetyltransferase with a peptide substrate in a presence of a test compound,

(b) detecting an amount of an acetylated peptide substrate using an anti-acetylated peptide antibody, wherein the anti-acetylated peptide antibody recognizes only an acetylated form of the peptide substrate and does not recognize to any appreciable degree the peptide substrate in its unacetylated form,

(c) comparing the amount of the acetylated peptide substrate detected in step (b) with a control amount defined as an amount of an acetylated peptide substrate detected in an absence of the test compound, and

(d) selecting the compound associated with an increase or decrease in the amount of the acetylated peptide substrate as compared to the control amount.

23. A kit for the screening method of claim 16 [23], comprising an anti-acetylated antibody.

rev: January 3, 2002

Appendix A

HOME (index page)

Return (alphabetical antibody index page)

Return (tumor marker related ab index page)

ANTIBODIES

(anti-Human and others as indicated)

Research Diagnostics Inc offers a wide line of antibodies. Since no one antibody works best for all applications (neutralization, blotting, histochemistry, ELISA, etc), we offer many different types of antibodies to help solve this probl m. Please inquire for other applications or types of antibodies not listed below.

MONOCLONAL ANTIBODIES TO p53 Oncoprotein:

-FOR RESEARCH USE ONLY :

available unlabelled or labelled as indicated -FT=FITC labeled -BT-biotin labeled

custom conjugates may be available please inquire clone **=reactive on formalin fixed paraafin sections

see individual clone specs below chart

cat#	description	clone	isotype	size	
RDI-CBL773	mouse anti-P53 human (aa250-270)	DO-12	mIgG2b	200ug/2ml	$300.00
RDI-CBL774	mouse anti-p53 human (56-65)	DO-14 **	mIgG1	200ug/2ml	$300.00
RDI-CBL423	mouse anti-p53 human C term (aa370-378)	Pab122	mIgG2b	200ug/2ml	$300.00
RDI-CBL423BT	mouse anti-p53 human C term:Biotin (aa370-378)	Pab122	mIgG2b	100tests	$300.00
RDI-CBL423FT	mouse anti-p53 human C term:FITC (aa370-378)	Pab122	mIgG2b	100tests	$300.00
RDI-CBL429	mouse anti-p53 human C term (aa371-380)	Pab421	mIgG2a	200ug/2ml	$300.00
RDI-CBL429BT	mouse anti-p53 human C term:Biotin (aa371-380)	Pab421	mIgG2a	100 tests	$300.00
RDI-CBL429FT	mouse anti-p53 human C term:FITC (aa371-380)	Pab421	mIgG2a	100 tests	$300.00
RDI-CBL422	mouse anti-p53 human N term (aa20-25)	BP53-12 **	mIgG2a	200ug/2ml	$300.00
RDI-CBL422BT	mouse anti-p53 human N term:Biotin (aa20-25)	BP53-12	mIgG2a	100 tests	$300.00
RDI-CBL422FT	mouse anti-p53 human N term:FITC (aa20-25)	BP53-12	mIgG2a	100 tests	$300.00
RDI-CBL420	mouse anti-p53 human N term (aa32-79)	Pab1801 **	mIgG1	200ug/2ml	$300.00
RDI-CBL420FT	mouse anti-p53 human N term:FITC (aa20-25)	Pab1801	mIgG1	100 tests	$300.00
RDI-CBL404	mouse anti-p53 human (aa213-217)	Pab240	mIgG1	200ug/2ml	$300.00

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Cytoplasmically "Sequestered" Wild Type p53 Protein Is Resistant to Mdm2-mediated Degradation*

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The Mdm2 oncoprotein mediates p53 degradation at cytoplasmic proteasomes and is the principal regulator for maintaining low, often undetectable levels of p53 in unstressed cells. However, a subset of human tumors including neuroblastoma constitutively harbor high levels of wild type p53 protein localized to the cytoplasm. Here we show that the abnormal p53 accumulation in such cells is due to a profound resistance to Mdm2-mediated degradation. Overexpression of Mdm2 in neuroblastoma (NB)[1] cell lines failed to decrease the high steady state levels of endogenous p53. Moreover, exogenous p53, when introduced into these cells, was also resistant to Mdm2-directed degradation. This resistance is not due to a lack of Mdm2 expression in NB cells or a lack of p53-Mdm2 interaction, nor is it due to a deficiency in the ubiquitination state of p53 or proteasome dysfunction. Instead, Mdm2-resistant p53 from NB cells is associated with covalent modification of p53 and masking of the modification-sensitive PAb 421 epitope. This system provides evidence for an important level of regulation of Mdm2-directed p53 destruction *in vivo* that is linked to p53 modification.

Controlling the stability of the p53 tumor suppressor protein is crucial for an effective cellular stress response when needed and for keeping this dangerous molecule in check when not needed. p53 levels are largely regulated by interaction with Mdm2, a negative regulator of p53 and the product of a p53-inducible gene (1, 2). Posttranslational modification has been associated with stabilizing p53 protein during cellular stress responses. DNA damage induces specific phosphorylations on N-terminal serine residues of p53 *in vivo*, possibly catalyzed by various stress kinases, thereby preventing Mdm2 binding, which in turn alleviates transcriptional inhibition and stabilizes p53 by inhibiting its degradation (3, 4). As a consequence, p53 half-life prolongs from minutes to hours (5). Conversely, tight regulation of p53 is critical for normal growth of unstressed cells, in which p53 is a short lived protein (half-life, 20–30 min) that is maintained at low, often undetectable levels through continuous degradation mainly directed by Mdm2. Hence, Mdm2 is largely responsible for the high p53 turnover in undamaged cells (6). Mdm2-mediated p53 degradation occurs through a ubiquitin-dependent pathway on cytoplasmic 26S proteasomes (7, 8). Mdm2 functions as a p53-specific E3 ubiquitin ligase *in vitro* (9) and this ubiquitination probably takes place in the nucleus on the large p300/CBP protein, serving as a scaffolding (10).

Recent studies of p53 turnover using overexpression assays with p53 and Mdm2 mutants elucidated some structural requirements for Mdm2-dependent destruction of p53. Together, these studies reveal the importance of several regions on both proteins, particularly on the N and C termini, the precise contributions of which, however, are not all understood. A basic but insufficient requirement is a direct interaction between the two proteins through their N termini. An Mdm2-binding site mutant of p53 (conserved box I) is resistant to degradation by Mdm2 (1, 2). Crystallographic analysis of the interacting domains has shown a tight key-lock configuration (11), with p53 domain amino acids 17–27 fitting deeply into a hydrophobic cleft of Mdm2. Also, Mdm2 shuttles between the nucleus and the cytoplasm, and Mdm2-directed degradation of p53 depends on a functional nuclear export signal of Mdm2 (12–14). Lastly, the C-terminal RING finger domain of Mdm2 is somehow required, because deletion or mutations in this region act as dominant negative mutants and protect p53 from degradation by endogenous Mdm2 (15). In addition, the p14ARF protein, which binds to the C-terminal region of Mdm2, can inhibit Mdm2-mediated p53 degradation without disrupting the p53-Mdm2 complex (16–18), possibly due to inhibiting the E3 ubiquitin ligase activity of Mdm2 (19). To date, known requirements on p53 include p53 tetramerization, which, although not absolutely required, enhances degradability, possibly via improved Mdm2 binding. A monomeric p53 mutant lost sensitivity to degradation by Mdm2 (20). Also, the extreme C terminus of p53 (amino acids 363–393) is required, because p53 mutants with deletions of this region show constitutive Mdm2 resistance in unstressed cells due to an unknown mechanism (20).

Certain human tumors, including neuroblastoma (21), breast cancer (22–24), colon cancer (25–27), and retinoblastoma (28), as well as normal mouse embryonic stem cells (29), constitutively accumulate high levels of wild type p53 protein in their cytoplasm in the absence of stress. This is due to a dramatic increase in p53 half-life (>8 h in unstressed neuroblastoma cells) (30), thereby stabilizing the protein. Thus, this phenotype is due to inefficient degradation of p53 rather than to increased synthesis. The cytoplasmic p53 accumulation is also the hallmark of a concomitant defect in p53 function in response to genotoxic stress (31, 32, 29) and, in fact, prompted its original observation (21, 22). We recently showed that this seemingly static sequestration of p53 is in fact not due to a blocked nuclear entry but due to a dynamic imbalance characterized by hyperactive p53 export from the nucleus (33). Functional inactivation of p53 in response to stress is therefore to a large degree due to an inefficiency in nuclear retention of p53 (31, 32, 29). Using human neuroblastoma cell lines, we show here that the aberrant constitutive p53 accumulation in these

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[1] The abbreviations used are: NB, neuroblastoma; GFP, green fluorescent protein; CBP, cAMP response element-binding protein.

cells is due to resistance to Mdm2-mediated degradation. This resistance is observed despite normal levels of Mdm2 protein, p53-Mdm2 complexes, and p53 ubiquitination. Instead, stabilization correlates with covalent modification of p53, characterized by an acidic shift in the charge isoform profile of p53 and masking of its modification-sensitive 421 epitope. This system provides evidence for a novel level of *in vivo* regulation of p53 destruction by Mdm2 linked to posttranslational p53 modification.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The following cell lines were of human origin: the neuroblastoma lines LAN-5, SK-N-SH, IMR 32, CHP 134, and SK-N-AS exhibit constitutive cytoplasmic accumulation of wild type p53 protein. The osteosarcoma line SaOs-2 is homozygously deleted for p53; the neuroepithelioma line CHP 100 is deficient for p53 expression due to aberrant mRNA; the chronic myelogenous leukemia line ML-1, the fibrosarcoma line HT 1080, and the diploid immortal fibroblast line IMR 90 all contain low levels of functional wild type p53; and the colon carcinoma line RKO contains mildly elevated levels of functional wild type p53. The breast cancer lines MDA 231 and MDA 468 harbor a R280K and R273K mutation, respectively. Mouse DM cells harbor an amplification of the Mdm2 gene (34). All cells were cultured in Dulbecco's modified Eagle's medium/10% fetal calf serum. For p53 ubiquitination, proteasome inhibitor MG101 (50 mM) (Sigma) was added to the culture medium for 5 h before lysates were prepared. Baculoviral human wild type p53 protein was purified on a MonoQ column and appeared as a single band on silver gels. Various normal human tissues were collected at University Hospital Stony Brook during surgical resections and immediately snap frozen after harvesting. Undifferentiated neuroblastoma tumors were previously described and collected in the same way (21).

Plasmids—Human Mdm2 expression plasmids, all pCMV BamNeo-based (35), were kindly provided by Arnold J. Levine. HDM2 encodes wild type Mdm2 (36), G58A encodes a contact mutant of Mdm2 that abolishes its interaction with p53 (8), and mutant nuclear export signal encodes an export mutant of Mdm2, leading to its nuclear retention due to the change of two critical hydrophobic residues within the nuclear export signal (L205A and I208A) (12). N-terminally FLAG-tagged human wild type p53 (Fwtp53) was generated by polymerase chain reaction using pC53-SN3 (35) as template. The sense primer contained a *Bam*HI site upstream of the sequence encoding the FLAG octapeptide (5′-AG CAG TTG GGA TCC ATG GAC TAC AAG GAC GAC GAT GAC AAG ATG GAG GAG CCG CAG TCA GAT CCT AGC G-3′), and the antisense primer also contained a *Bam*HI site (5′-TTA TTC GGA TCC AGA ATG TCA GTC TGA GTC AGG CCC-3′). The polymerase chain reaction product was cloned into pCMV BamNeo. The pFwtp53 plasmid was also used to construct the p53 C-terminal plasmids F305–360 and F320–360 by polymerase chain reaction overlap extension technique as described (33). The plasmid pcDNA3-Myc-ARF expressing human wild type p14ARF fused to a Myc tag was described previously (37). Green fluorescent protein (GFP) expression plasmid (CLONTECH) was co-transfected in transient transfections to normalize relative transfection efficiency.

Transfections—Cells were plated in 60-mm dishes and grown overnight to 80% confluence. Two mg of Mdm2 wild type or mutant-encoding plasmid was co-transfected with 0.4 mg of GFP-encoding plasmid and 0.6 mg of wild type p53-encoding plasmid or CMV BamNeo empty vector using the LipofectAMINE Plus reagent (Life Technologies, Inc.) as recommended by the manufacturer. For transient transfections, cells were collected after 24 h, whereas stable transformants were selected by growth for 21 days in medium containing 0.5 mg/ml G418 (Life Technologies, Inc.), ring cloned, and expanded into single cell clones. For Myc-p14ARF expression, 2 mg of plasmid was used.

Antibodies, Western Analysis, and Immunoprecipitation—Cell lysates from unstressed cells were prepared as described (31), subjected to 8% SDS-polyacrylamide gel electrophoresis, and transferred to nylon membranes. Immunoblots were visualized by ECL (Pierce). Cell lysates (1 mg of total protein) were immunoprecipitated with 1.5 μg of the indicated antibody, incubated with 30 μl of protein G-agarose beads (Life Technologies, Inc.), washed five times in radioimmune precipitation buffer (50 mM Tris, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, pH 7.4) and visualized as described (31). Antibodies to p53 were monoclonals PAb 421, 1801, and DO-1 (Oncogene Science), which recognize epitopes amino acids 372–382 (421), 46–55 (1801), and 20–25 (DO1). CM-1 is a polyclonal rabbit antibody raised against bacterial recombinant human wild type p53 (Vector). FLAG-tagged p53 constructs were detected with M2 (Eastman Kodak Co.). Mdm2 was detected with monoclonal IF2 (Oncogene Science). Other antibodies used were specific for vimentin (BioGenex), GFP (CLONTECH), Myc (NeoMarker), IkBa (Santa Cruz), actin (Sigma), mouse IgG (Sigma), and Lamin A (Chemicon).

Immune-isoelectric Focusing—Crude cell lysates (2–20 mg), prepared by sonication without ionic detergents and low salt (37), were mixed with equal volume of 2× sample buffer (4.8 g urea, 120 ml each of ampholytes pH 4–6 and 5–7 (Bio-Lyte, Bio-Rad), 1 ml of 20% Triton X-100, 100 ml of 2-mercaptoethanol, 1 mg of bromphenol blue in 10 ml of H_2O) 5 min before loading. Loaded lysates and pH markers (Bio-Rad) were overlaid with 1% mixed ampholytes and 5% sucrose to protect proteins from the harsh pH conditions of the upper chamber. Proteins were resolved along a gradient of pH 7–4 on one-dimensional slab mini gels using polyacrylamide containing 8 M urea and 120 ml each of ampholyte pH 4–6 and pH 5–7 as described (38). The upper chamber buffer (500 ml of catholyte, 20 mM sodium hydroxide) and lower chamber buffer (500 ml of anolyte, 10 mM phosphoric acid) were prepared fresh and degassed, and gels were run at 150 V for 30 min followed by 200 V for 3 h. Gels were transferred to nylon membranes and p53 was detected by immunoblotting with DO-1. In parallel, equal aliquots were run on 8% SDS-polyacrylamide gel electrophoresis gels followed by DO-1 immunoblotting to verify p53 loading.

Immunofluorescence—Subconfluent cells in P100 dishes were refed 4 h prior to calcium phosphate-mediated transfection with 20 mg each of p53 C-terminal peptide or empty vector DNA constructs. After overnight incubation, cells were aliquoted into polylysine-coated chamber culture slides (Becton Dickinson) and grown for an additional 24 h. Cells were immunostained as described (33) and examined with a Nikon scanning laser microscope. Expression of constructs was verified by FLAG antibodies and was reproducible with transfection efficiency over 50% (data not shown).

RESULTS

No Deficiency of Endogenous Mdm2 Protein in Neuroblastoma Cells—To exclude the possibility that the constitutively high levels of wild type p53 protein in neuroblastoma (NB) cells are due to a lack of expression of its destabilizer Mdm2, we compared a panel of NB cells (SK-N-SH, LAN-5, IMR 32, CHP 134 and SK-N-AS) with a broad range of other human cell lines (Fig. 1*A* and data not shown). These made up four different types of p53 steady state levels, including p53-deficient cells (SaOs-2 and CHP 100), low levels of functional p53 (HT 1080, IMR 90, and ML-1), mildly elevated levels of functional p53 (RKO), and markedly increased levels of mutant p53 due to a failure to induce Mdm2 (MDA 231 and 468). Fig. 1*A* shows that steady state levels of Mdm2 protein in NB cells are comparable to Mdm2 levels in all other cell lines analyzed, with some variations in expression in both categories. The only exception was an undetectably low Mdm2 level in ML-1 cells (not shown) for reasons that are not clear.

Mdm2 Interacts with p53 in NB Cells—A second possibility that could explain the abnormal stability of p53 would be a lack of interaction between p53 and Mdm2. To test this, we performed co-immunoprecipitation assays from LAN-5 cells with an Mdm2-specific antibody. Fig. 1*B* shows the presence of p53-Mdm2 complexes indicating their interaction *in vivo* (*lanes SaOs-2* and *RKO*). Moreover, the amount of complexed p53 in LAN-5 cells is similar to the amount in IMR 90 control cells (compare lanes *RKO* and *HT1080*). IMR 90 fibroblasts contain low levels of functional p53, indicating sensitivity to Mdm2-directed degradation. These data demonstrate that the abnormal p53 stability in NB cells is not due to a lack of p53-Mdm2 interaction. To further demonstrate that Mdm2 function is normal in NB cells, we tested its ability to enter the physiologically important interaction with p14ARF. Fig. 1*C* shows readily demonstrable *in vivo* complexes between Mdm2 and p14ARF, co-immunoprecipitated from a stably expressing Mdm2 NB cell line (clone HDM2–2 of Fig. 2*D*) and transiently transfected with Myc-tagged p14ARF.

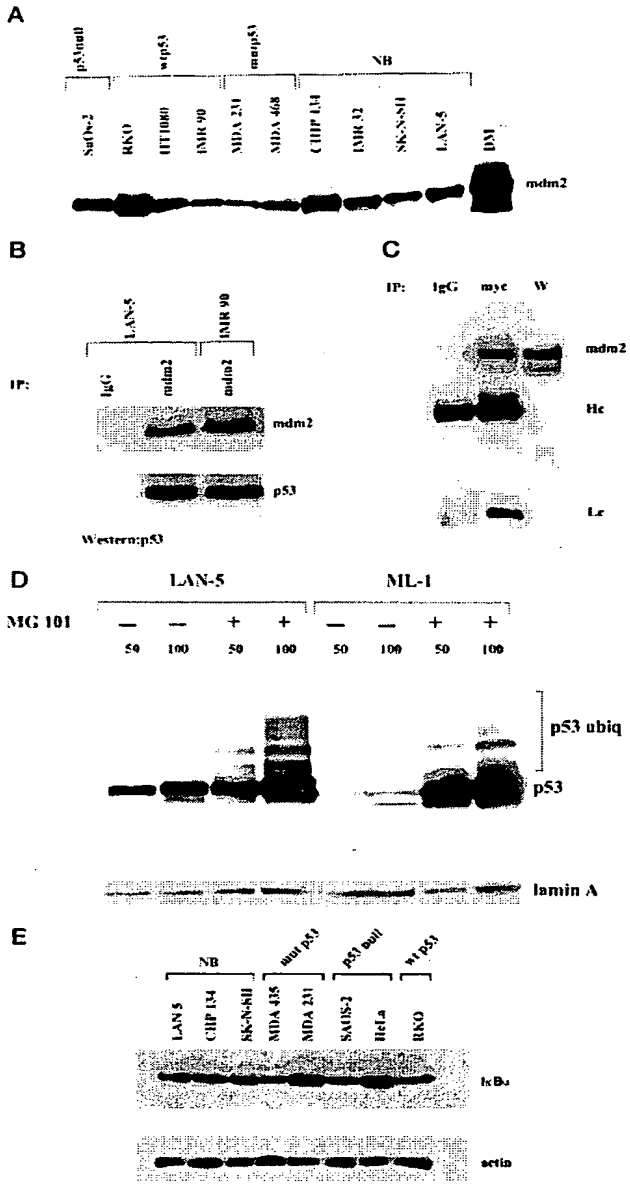

FIG. 1. **Abnormal p53 stability in neuroblastoma cells is not due to a deficiency of Mdm2, lack of p53-Mdm2 interaction, or lack of p53 ubiquitination.** *A*, Mdm2 protein levels in NB cells are within normal range. NB cell lines were compared with a broad array of other cell lines. These fall into 4 categories of p53 steady state levels including p53-deficient cells (SaOs-2), low levels of functional p53 (HT 1080 and IMR 90), mildly elevated levels of functional p53 (RKO), and markedly increased levels of mutant p53 due to a failure to induce Mdm2 (MDA 231 and 468). Lysates (100 mg) were subjected to immunoblot analysis with anti-Mdm2 (IF2). DM cells are mouse 3T3 fibroblasts with amplification of the Mdm2 gene and are shown for comparison. *B*, Mdm2 interacts with p53. Lysates (1 mg) of LAN-5 and IMR 90 cells were subjected to immunoprecipitation with anti-Mdm2 (IF2) or mouse IgG (1.5 mg each) and co-precipitated p53 was detected with CM-1. Loading of the blot was normalized for equal intensities of p53 bands between the two cell lines. *C*, Mdm2 in NB cells retains its ability to enter into complexes with a major physiologic partner, p14ARF. Clone HDM2–2 (see Fig. 2*D*) was transiently transfected with a Myc-p14ARF expression plasmid. Lysates (1 mg) were immunoprecipitated (*IP*) with 2 μg of anti-Myc or mouse IgG and immunoblotted with anti-Mdm2. *Lane W* represents an immunoblot only. *D*, p53 is properly ubiquitinated. Lysates (50 or 100 μg) from LAN-5 and ML-1 cells grown in the absence (−) of presence (+) of proteasome inhibitor MG 101 (50 mM) for 5 h were subjected to immunoblot analysis with anti-p53 DO-1 and anti-lamin A (loading control). *E*, neuroblastoma cells have a functional ubiquitin-proteasome pathway. Steady state levels of IkBa in NB cell lines are comparable to those from a broad range of non-NB tumor lines. Shown is an immunoblot (100 μg of lysates) with anti-IkBa and anti-actin as loading control.

p53 from NB Cells Is Properly Ubiquitinated—Next we examined whether p53 ubiquitination in NB cells is deficient, because poor ubiquitination of p53 could lead to its stabilization. Also, Mdm2 is a p53-specific E3 ubiquitin ligase *in vitro*. LAN-5 cells and ML-1 control cells, which contain low levels of p53 indicating Mdm2 sensitivity, were treated with the proteasome inhibitor MG101 for 5 h. p53 stabilized in both cases to a similar degree (Fig. 1*C*, *top panel, lowest band*). More importantly, the ladder of polyubiquitinated p53 species were similar in LAN-5 and ML-1 cells (Fig. 1*D*). This indicates that the *in vivo* ubiquitination of p53, whether catalyzed by Mdm2 or some other E3 ligase, is functional in NB cells. Furthermore, these data also show that NB cells do not have a global functional defect of their proteasomes in processing ubiquitin-tagged proteins, because if this were the case, ubiquitinated p53 should have been easily detected even in untreated cells. To further confirm this point, we asked whether the processing of other proteasome-degraded proteins is normal in NB cells. IkBa, the cytoplasmic inhibitor of NFkB, is a prototype protein undergoing ubiquitin-mediated proteasome degradation (39). Fig. 1*E* shows that IkBa levels in NB cells are completely within the range of non-NB cells, indicating normal ubiquitin/proteasome processing in NB cells. Likewise, the same reasoning holds for Mdm2, the levels of which in NB cells are within normal range, as shown above (Fig. 1*A*), because Mdm2 itself is degraded via the ubiquitin proteasome pathway (40).

p53 Is Resistant to Ectopic Mdm2 in NB Cells—Although endogenous Mdm2 in NB cells is sufficiently expressed and enters into complexes with p53, it still left the possibility that Mdm2 had a specific defect in directing p53 destruction. We therefore asked whether the abnormal p53 stability could be overcome by overexpression of functional ectopic Mdm2 protein. SaOs-2 control cells (p53 null), transiently transfected with human wild type p53 (Fig. 2*A*, *lane 2*), showed a dramatic decrease in the endogenous p53 protein level when co-transfected with wild type Mdm2 (*lane 3*) but not with a p53-contact mutant of Mdm2 (G58A) (*lane 4*) as described previously (1, 2, 12). In contrast, endogenous p53 protein from LAN-5 (Fig. 2*B*) and SK-N-SH (Fig. 2*C*) was completely resistant to degradation by forced expression of wild type Mdm2 (Fig. 2, *B* and *C*, compare *lanes 1* and *2* with *lane 3*), as well as to a nuclear export mutant of Mdm2 (Fig. 2, *B* and *C*, *lane 4*) and to a contact mutant of Mdm2 (Fig. 2, *B* and *C*, *lane 5*). To confirm this result, we generated a series of stable LAN-5 subclones that overexpress either vector alone, wild type Mdm2, or contact mutant Mdm2. Of six wild type Mdm2 clones, all showed marked resistance of p53 protein toward degradation (Fig. 2*D*, compare *lanes 2–5* with *lanes 1, 6,* and *7*). Taken together, the data demonstrate that the abnormal stability of p53 in NB cells is due to a profound resistance of the p53 protein to Mdm2-directed degradation and strongly suggest that the principal defect lies in p53 and not in Mdm2.

To further support this conclusion, we asked whether exogenous human wild type p53, when transfected into NB cells, was also resistant to overexpressed Mdm2. Fig. 3 shows that this is indeed the case. FLAG-tagged p53 (Fwtp53), when transiently transfected into SK-N-SH and LAN-5 cells, is markedly resistant to co-transfected wild type Mdm2, as it is resistant to

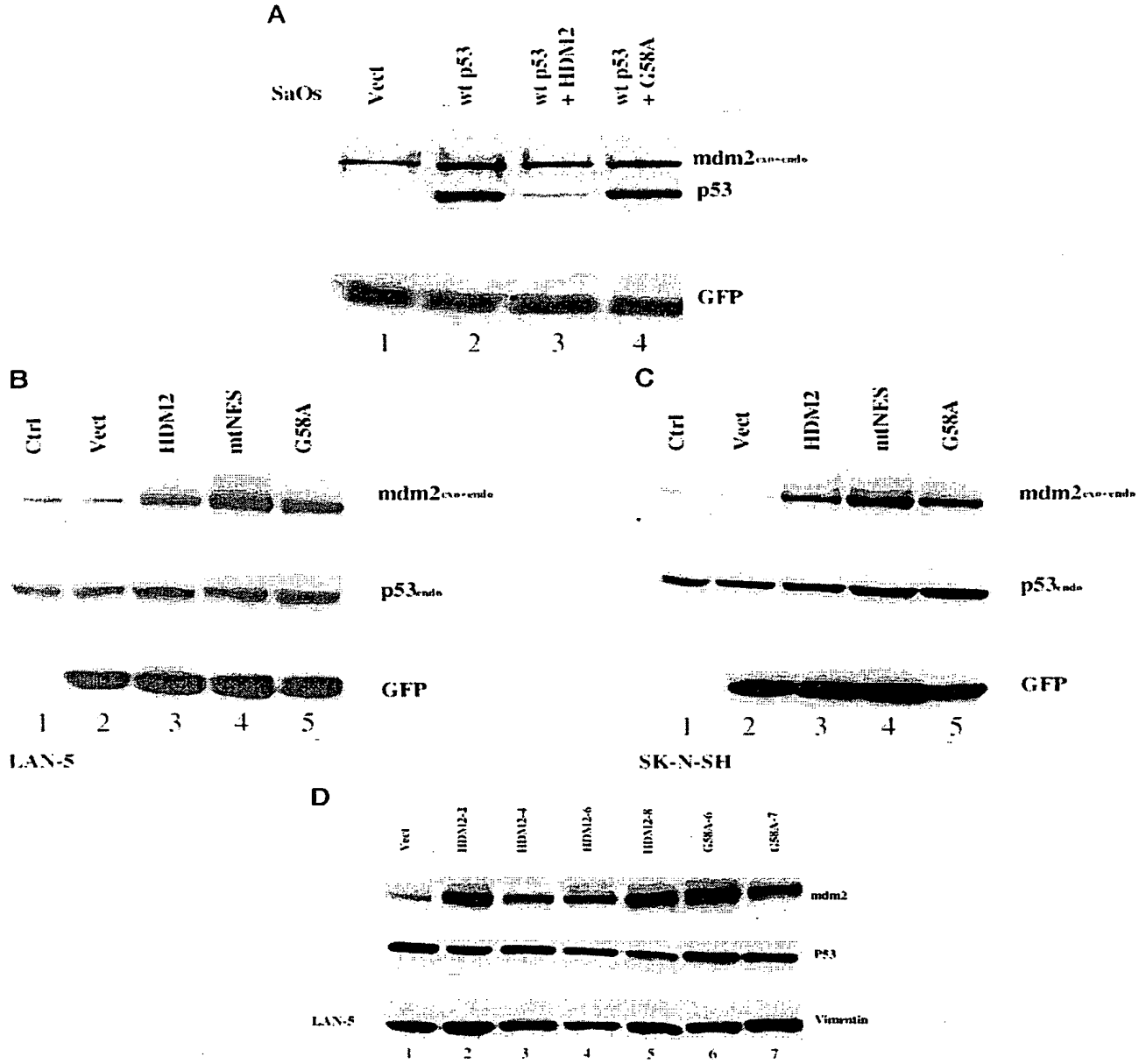

FIG. 2. **Endogenous p53 is resistant to exogenous Mdm2 in NB cells.** SaOs2 (*A*), LAN-5 (*B*), and SK-N-SH (*C*) cells were transiently transfected with either wild type Mdm2 (HDM2), nuclear export mutant Mdm2 (mutant nuclear export signal (*mtNES*)), p53-contact mutant Mdm2 (G58A) or empty vector (*vect*). p53-deficient SaOs2 control cells were also co-transfected with wild type p53. GFP was co-transfected in all cases to normalize the expression. *D*, a series of stable LAN-5 subclones that overexpress either empty vector, wild type Mdm2 (HDM2 clones), or p53-contact mutant Mdm2 (G58A clones). Lysates were subjected to immunoblot analysis with anti-Mdm2 (IF2), anti-p53 (DO-1), anti-GFP, and anti-vimentin (loading control).

contact mutant Mdm2 (Fig. 3, compare *lane 2* with *lane 3* and *4*). Together, these data show that the cellular environment of NB cells causes the loss of sensitivity of the p53 protein toward Mdm2-regulated turnover.

p53 Resistance to Mdm2-directed Degradation Is Associated with Covalent Modification of p53—Because the above results imply the p53 protein itself in mediating its degradation defect, we investigated whether wild type p53 protein from NB cells is constitutively subject to altered posttranslational modification. Immune-isoelectric focusing is a commonly used technique to characterize charge isoform profiles of proteins. The high urea content used in the polyacrylamide gels (8 M) ensures the elimination of protein-protein interactions as another potential source of charge alterations. Cell lysates from SK-N-SH and LAN-5 reveal an identical and specific shift in their p53 charge isoform profiles when compared with control p53 proteins derived from cellular (MDA 231) or baculoviral sources (Fig. 4*A*, *left panel*). Both NB lines show a shift in profile toward four prominent hyperacidic isoforms with isoelectric points (pI) ranging from about 4.6 to 5.3 (*20 μg SH* and *15 μg LAN-5*). In contrast, both control p53 proteins exhibit inverted profiles (in relative proportions of the individual species) that are domi-

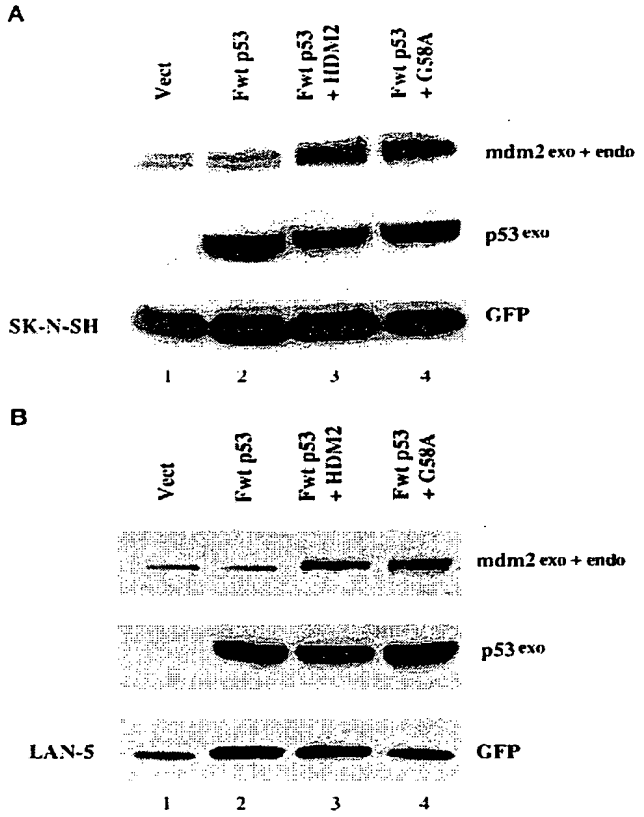

FIG. 3. **Exogenous p53 is equally resistant to exogenous Mdm2 in NB cells.** SK-N-SH (*A*) and LAN-5 (*B*) cells were transiently transfected with FLAG-wild type p53 fusion protein (*lanes 2–4*), and either empty vector, wild type Mdm2 (HDM2), or contact mutant Mdm2 (G58A). *Lane 1* contains empty vector (*vect*) only. GFP was co-transfected in all cases to normalize the expression. Lysates were subjected to immunoblot analysis with anti-Mdm2, anti-FLAG, and anti-GFP.

nated by more basic isoforms (*2 μg MDA 231* and *60 ng bac p53*). Purified baculoviral human wild type p53 (*60 ng bac p53*) has been shown to exhibit identical posttranslational modifications to normal cellular p53 (41). Mutant p53 from MDA 231 cells (*2 μg MDA 231*) harbors a charge-neutral R280K exchange with loss of transactivating function. MDA 231-derived p53 was chosen to show that p53 modification is not a consequence of abnormal stability (which in this case is caused by the inability of mutant p53 to induce Mdm2). Both profiles consist of several tightly packed coalescing isoforms with pIs ranging from 6 to 6.5, whereas the hyperacidic species so characteristic for NB cells make up only a minor fraction. Conversely, the presence of the basic species in NB cells was not consistent and, even when present, presented only minor fractions. Supporting these data, on SDS-polyacrylamide gel electrophoresis gels, neuroblastoma tumors and NB cell lines exhibit two p53 bands after immunoprecipitation, which are best seen with the polyclonal CM-1 antibody. Of these, the dominant p53 species runs slower than the faster migrating single p53 species present in normal human tissues and ML-1 cells (Fig. 4*B*, compare *lanes 5–7* with *lanes 2–4*, and data not shown). The observed modifications are consistent with aberrant phosphorylation and/or acetylation or other less common acidifying modifications. rRNA moieties, covalently bound to a small subset of p53 polypeptides on ribosomes of normal cells in the G_1 phase of the cell cycle (42), are excluded because RNase

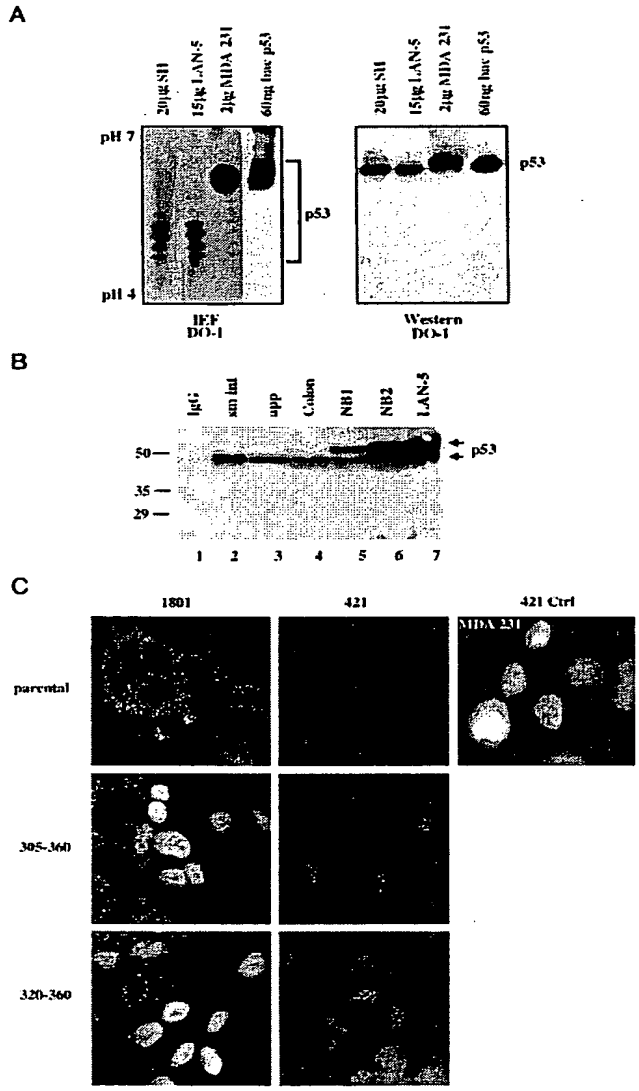

FIG. 4. **p53 resistance toward Mdm2-directed degradation is associated with covalent modification of p53.** *A, left panel,* one-dimensional immune isoelectric focusing (*IEF*) of lysates from SK-N-SH (*SH*), LAN-5, and MDA 231 breast carcinoma cells. *Right lane* contains highly purified baculoviral human wild type p53 protein. The pH gradient is indicated on the *left*. *Right panel,* identical aliquots were immunoblotted to verify comparable loading. Both panels were probed with anti-p53 (DO-1). *B,* immunoprecipitation of p53 from small intestine (*lane 2*), appendix (*lane 3*), colon (*lane 4*), two undifferentiated neuroblastomas (*lanes 5* and *6*) and LAN-5 (*lane 7*) with CM-1 followed by DO-1 blotting. *Lane 1* contains no lysate. *C,* the 421 epitope of degradation-resistant p53 is masked independently of its subcellular localization. LAN-5 cells were transiently transfected with p53 C-terminal peptides 305–360 and 320–360. These peptides span the tetramerization domain and cause nuclear retention of endogenous p53 due to heterooligomerization and interference with hyperactive nuclear export. Shown is the immunofluorescence of parental and transfectant cells with PAB 1801 (*left column*) and 421 (*right column*). Both antibodies are specific for endogenous p53. PAb 421 is a modification-sensitive antibody that recognizes its epitope (amino acids 372–382) in the unmodified state. Whereas PAb 1801 recognizes p53 in the cytoplasmic (parental) and nuclear (transfectant) compartment of LAN-5 cells, PAb 421 gives no signal or only a minimal signal. MDA 231 control cells are well recognized by 421. × 400.

A pretreatment of the NB lysates does not change the isoform profile (data not shown).

To further support the above findings, we probed NB cells with the p53 monoclonal antibody PAb 421, the ability of which to recognize its epitope (amino acids 372–382) is modification-dependent (43–46). We had previously observed in immunofluorescence assays that the 421 epitope in NB cells with cytoplasmically sequestered p53 is completely masked (47). We then asked whether this masking was dependent on cytoplasmic localization. To this end, we exploited the effect of co-expressed p53 C-terminal polypeptides. These heterooligomerizing peptides specifically cause nuclear retention of endogenous p53 in NB cells by disrupting hyperactive nuclear export, probably through a combination of burying the intrinsic nuclear export signal of p53 and interfering with its Mdm2-mediated export (33). This effect is not due to a nonspecific titration of CRM1 export receptors, because the localization of IkBa, a marker shuttling protein, is unaffected by the C-terminal peptides (33). Transient transfections with plasmids encoding the p53 polypeptides 305–360 and 320–360 show that the 421 epitope remains largely unrecognizable despite nuclear translocation of p53 (Fig. 4*C*, compare *left* and *right panels*). Both PAb 1801 and PAb 421 antibodies are specific for endogenous p53. Yet, whereas the 1801 antibody gives a very strong signal, the 421 signal is barely detectable. This clearly shows that the 421 epitope of NB p53 is masked constitutively and independently of its subcellular localization. These data further support covalent modification of p53 that includes the amino acid 372–382 region.

DISCUSSION

In this study we investigated the mechanism responsible for the abnormal stability of wild type p53 protein, which constitutively accumulates in the cytoplasm of certain tumor cells and embryonic stem cells. Concomitantly, cytoplasmic p53 accumulation in such cells is also the hallmark of a defect in p53 function in response to genotoxic stress and oncogenic transformation (31, 32, 29, 21, 22). We recently showed that the seemingly static sequestration of p53 is due to hyperactive export of p53 from the nucleus (33). Functional inactivation of p53 in response to stress is therefore promoted by an inefficiency in nuclear retention of p53 (31, 32, 29).

Using transient and stable overexpression assays in neuroblastoma lines, we show here that the abnormal p53 stability of the cytoplasmic sequestration phenotype is due to a profound resistance of p53 toward Mdm2-mediated degradation. Moreover, we find that degradation-resistant p53 is associated with an altered posttranslational modification profile of the protein. This alteration is characterized by loss of positively charged moieties and/or gain of negatively charged moieties that include the amino acid 372–382 region. The fact that the p53 isoform profile in NB cells is inverted rather than completely novel compared with p53 from other sources suggests that the same set of p53 modifying enzymes that are usually at work are active in NB cells but that their relative activities are grossly altered. Although aberrant phosphorylation is a possibility, our attempts to demonstrate it by pretreating immunoprecipitated p53 with phosphatases have so far been unconvincing. Future work will focus on the precise identification of sites and types of modifications in NB p53. The observed 421 epitope masking, independent of subcellular localization, is completely consistent with modification of the amino acid 372–382 region or the surrounding area. Furthermore, it could be structurally akin to the effect seen with p53 delta 30 deletion, which renders the protein Mdm2-resistant (20). Examples of complete 421 masking have been described for epitope glycosylation *in vivo* (40), protein kinase C-mediated phosphorylation of Ser-378 *in vitro* (44), and partially for p300/CBP-mediated acetylation of Lys-373 and Lys-382 *in vitro* (46). In contrast to NB cells, however, select phosphorylation or acetylation of recombinant p53 *in vitro* correlates with activation of p53 sequence-specific DNA binding (44, 46). This reinforces the notion that it is the specific modification profile of p53 in any given case that determines its effects on stability and function of the protein.

A number of reasons support the conclusion that the cause for the abnormal p53 stability in the cytoplasmic sequestration phenotype lies in p53 itself. First, the loss of sensitivity is not due to a lack of Mdm2 expression or its ability to interact with p53, nor is it due to a defect in p53 ubiquitination or global proteasome dysfunction. Moreover, endogenous Mdm2 from NB cells is unlikely to be functionally defective in its p53-degrading activity because in that case active ectopic Mdm2 would have overcome the block. Alternatively, could there be a defect in the ability of Mdm2 to undergo nucleocytoplasmic shuttling, which was shown to be required for p53 degradation? Again, this is unlikely, given the hyperactive export of p53 that underlies its cytoplasmic sequestration. Also, a nuclear export mutant of Mdm2 (mutant nuclear export signal) failed to further increase the total cellular p53 levels in NB cells (Fig. 2, *B* and *C*). The cytoplasmic accumulation of p53 also makes it unlikely that an abnormality in p300/CBP, both of which are nuclear proteins, play a role here. The multifunctional p300 protein was shown to promote Mdm2-mediated p53 degradation *in vivo* through preformed p300-Mdm2 complexes, possibly by enabling the ubiquitin ligase activity of Mdm2 for p53 (10). In addition, we showed that p53 ubiquitination is not defective in NB cells.

During viral or cellular oncogene activation of the p53 pathway, p14ARF promotes p53 stabilization by inactivating Mdm2 (see Ref. 48 for review). As a possible mechanism, p14ARF inhibits the ubiquitin ligase activity of Mdm2 for p53 *in vitro* (19). During the attenuation of a p53 response, two antiparallel feed back loops connect p53 with its regulators. p53 up-regulates its destabilizer Mdm2 and down-regulates its activator p14ARF (18). In theory at least, the observed NB phenotype could be explained by constitutive overexpression or hyperactivity of p14ARF. However, p14ARF abnormalities in neuroblastoma have not been reported. Also, if this were the case, marked overexpression of Mdm2 in stable NB clones (Fig. 2*D*) should have overcome the p53 resistance to degradation.

Interestingly, we had no difficulties in generating stable wild type Mdm2 overexpressing neuroblastoma clones. This is in contrast to MCF-7 and U2OS cells, which do not tolerate stable overexpression of wild type Mdm2 (15), consistent with cell cycle arrest activities of the acidic domain of Mdm2 (49). Our experience suggests that this arrest ability of Mdm2 is cell type-dependent and that its absence might be linked to the p53 dysfunction present in NB cells. Cytoplasmic sequestration of p53 is the hallmark of its hyperactive nuclear export and prompted the original observation of this phenotype (21, 22). Yet, there is no *a priori* reason for p53 to accumulate in the cytoplasm, because its function might equally well be inactivated if p53 were immediately degraded after its export. This reasoning suggests a link between the two phenomena. p53 might be unable to lock itself efficiently into the nucleus in response to stress because of an aberrant modification that also prevents its efficient degradation by Mdm2. Interestingly, high levels of ectopic p53 (Fig. 3, *lanes 2*), which localized mainly to the nucleus, had either nil or only minimal activity in inducing transcriptional targets, as demonstrated by the lack of response of the endogenous Mdm2 gene (Fig. 3, *A* and *B*, compare *lanes 1* and *2*). Furthermore, endogenous and exogenous p53 in NB cells completely failed to induce apoptosis after DNA dam-

age.[2] These data further confirm the profound impairment of p53 function in NB cells. Taken together, the data suggest that hyperactive export of p53 in cells with a cytoplasmic phenotype is one of two mechanisms of p53 inactivation. The second mechanism is a functional block in the p53 signaling pathway, either on the level of p53 or downstream.

In summary, our results show the importance of proper posttranslational modification of the p53 protein in enabling high p53 turnover in resting cells. This level of regulation is distinct from the known structural requirements of the Mdm2 interaction site on the N terminus of p53 and additional C-terminal domains on both proteins. Interestingly, phosphorylation of the N or C terminus of p53 is not absolutely required for stress signal-induced stabilization of p53 (50). Conversely, aberrant p53 modification is clearly able to prevent Mdm2-mediated degradation in the absence of stress signals as shown here. Resistance to other regulators of p53 stability such as JNK may also play a role in NB cells (51). A full understanding of structural and regulatory requirements of Mdm2-mediated p53 destruction is critical, given the growing efforts in developing cancer agents directed at stabilizing wild type p53 through Mdm2 targeting, thereby activating p53 function (6, 52). Phenotypes with constitutive p53 accumulation, be it cytoplasmic or nuclear, will be valuable systems in elucidating important mechanisms that regulate p53 turnover.

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